

## $\alpha$ 1,3Fucosyltransferase VI is expressed in HepG2 cells and codistributed with $\beta$ 1,4galactosyltransferase I in the Golgi apparatus and monensin-induced swollen vesicles

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The major  $\alpha$ 1,3fucosyltransferase activity in plasma, liver, and kidney is related to fucosyltransferase VI which is encoded by the *FUT6* gene. Here we demonstrate the presence of  $\alpha$ 1,3fucosyltransferase VI ( $\alpha$ 3-FucT VI) in the human HepG2 hepatoma cell line by specific activity assays, detection of transcripts, and the use of specific antibodies. First, FucT activity in HepG2 cell lysates was shown to prefer sialyl-N-acetylactosamine as acceptor substrate indicating expression of  $\alpha$ 3-FucT VI. RT-PCR analysis further confirmed the exclusive presence of the  $\alpha$ 3-FucT VI transcripts among the five human  $\alpha$ 3-FucTs cloned to date.  $\alpha$ 3-FucT VI was colocalized with  $\beta$ 1,4galactosyltransferase I ( $\beta$ 4-GalT I) to the Golgi apparatus by dual confocal immunostaining. Pulse/chase analysis of metabolically labeled  $\alpha$ 3-FucT VI showed maturation of  $\alpha$ 3-FucT VI from the early 43 kDa form to the mature, endoglycosidase H-resistant form of 47 kDa which was detected after 2 h of chase.  $\alpha$ 3-FucT VI was released to the medium and accounted for 50% of overall cell-associated and released enzyme activity. Release occurred by proteolytical cleavage which produced a soluble form of 43 kDa. Monensin treatment segregated  $\alpha$ 3-FucT VI from the Golgi apparatus to swollen peripheral vesicles where it was colocalized with  $\beta$ 4-GalT I while  $\alpha$ 2,6(N)sialyltransferase remained associated with the Golgi apparatus. Both constitutive secretion of  $\alpha$ 3-FucT VI and its monensin-induced relocation to vesicles analogous to  $\beta$ 4-GalT I suggest a similar post-Golgi pathway of both  $\alpha$ 3-FucT VI and  $\beta$ 4-GalT I.

**Key words:** fucosyltransferase/human liver/monensin/Golgi apparatus

### Introduction

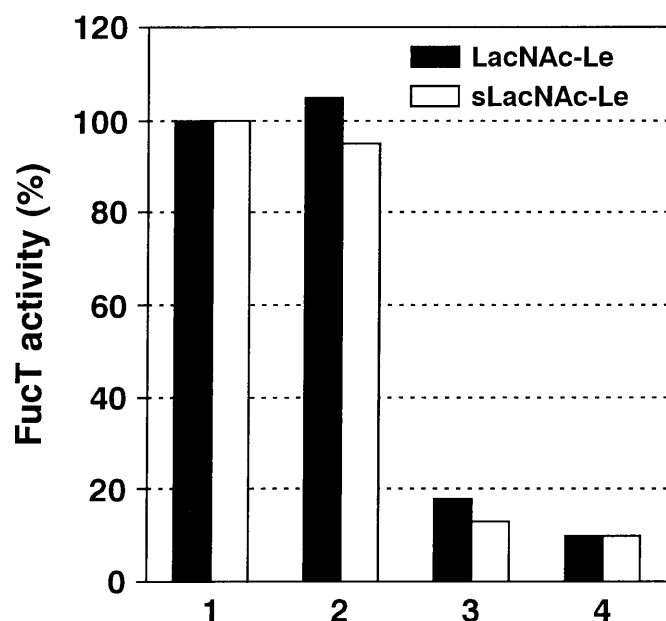
The  $\alpha$ 1,3fucosyltransferase ( $\alpha$ 3-FucT) enzyme activity has been detected in various types of tissues (Mollicone *et al.*,

1990, 1992; for review, see Macher *et al.*, 1991). FucTs constitute a family of homologous glycosyltransferases with a high degree of identity (Lowe, 1991). This family comprises five different fucosyltransferases, named  $\alpha$ 3-FucT III to VII (Goelz *et al.*, 1990; Kukowska-Latallo *et al.*, 1990; Koszdin and Bowen, 1992; Weston *et al.*, 1992a,b; Sasaki *et al.*, 1993; Natsuka *et al.*, 1994). These enzymes differ in their capacity to transfer fucose to distinct oligosaccharide acceptors, cation requirements and tissue specific expression (Goelz *et al.*, 1990; Kukowska-Latallo *et al.*, 1990; Mollicone *et al.*, 1990, 1992; Lowe, 1991; Macher *et al.*, 1991; Weston *et al.*, 1992a,b; Koszdin and Bowen, 1992; Sasaki *et al.*, 1993; Natsuka *et al.*, 1994). The five FucTs can be divided in two main subgroups, one comprising  $\alpha$ 3-FucTIV and VII, the other  $\alpha$ 3-FucTIII, V, and VI which are encoded by syntenically arranged genes. These are identical to 85% rendering their specific detection by molecular probes or immunological reagents difficult.

While the respective functions of  $\alpha$ 3-FucTIV and VII are being increasingly understood on the basis of their specific deletion in mice rendering them unable to synthesize selectin ligands (Lowe, 1997), the function of the three remaining FucTs needs to be further investigated. The study of developmental changes of tissue specific expression and the consequences of genetic polymorphisms all constitute approaches towards this goal (Mollicone *et al.*, 1994). For instance the *FUT5* and *FUT6* genes, respectively, encode two different enzymes with activities exhibiting the specificity of the plasma  $\alpha$ 1,3fucosyltransferases (Koszdin and Bowen, 1992; Weston *et al.*, 1992a). In several Java families the plasma  $\alpha$ 1,3fucosyltransferase activity has been found to be deficient. This deficiency was a result of a mutation in the *FUT6* gene (Mollicone *et al.*, 1994). The linkage relationship between a  $\alpha$ 3-FucT VI mutation and deficiency of plasma fucosyltransferase activity was further confirmed by work of van Dijk and associates (Brinkman-Van der Linden *et al.*, 1996): The missense mutation in the  $\alpha$ 3-FucT VI gene led to a complete absence of  $\alpha$ 3-fucosylation of serum glycoproteins. In sera of different individuals with inactivated *FUT6* gene but with a functional *FUT5* gene  $\alpha$ 1,3fucosyltransferase activity has not been detected (Brinkman-Van der Linden *et al.*, 1996). This finding excluded the involvement of *FUT5* in contributing to plasma FucT activity. While it seems clear that the *FUT6* gene encodes the plasma  $\alpha$ 1,3fucosyltransferase activity, its tissue origin has not formally been identified. Kidney is one candidate source of  $\alpha$ 3-FucT VI as a plasma fucosyltransferase activity. In the case of an  $\alpha$ 3-fucosylated individual with a congenital kidney anomaly, only 10% of FucT plasma activity has been detected which could be ascribed to the myeloid type (Caillard *et al.*, 1988). Lack of expression of the Le<sup>x</sup> antigen in

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**Fig. 1.** Inhibition of  $\alpha 1,3$ fucosyltransferase activity in HepG2 cell lysates using the OLI antiserum.  $\alpha 3$ -FucT VI activity was assayed in lysates of HepG2 cells as described in *Materials and methods* using LacNAc and sialyl-*N*-acetylglucosamine as acceptors, respectively. 1, Control assay mixture of 50  $\mu$ l supplemented with 20  $\mu$ l of H<sub>2</sub>O. 2, As in 1, with 20  $\mu$ l of preimmune serum (PIS). 3, As in 1, with 10  $\mu$ l of PIS and 10  $\mu$ l of OLI antiserum. 4, As in 1, with 20  $\mu$ l of OLI antiserum.

the kidney of this individual suggested that this organ may normally contribute to plasma FucT activity (Caillard *et al.*, 1988; Mollicone *et al.*, 1990). Another candidate for the origin of a plasma FucT activity is the liver, where transcripts of  $\alpha 3$ -FucT VI and enzyme activity corresponding to  $\alpha 3$ -FucT VI have been detected (Mollicone *et al.*, 1990, 1992; Johnson *et al.*, 1995). This organ is the source of many plasma proteins. In addition, another glycosyltransferase, e.g.  $\beta 1,4$ galactosyltransferase found in human serum originates at least partially from liver (Kim *et al.*, 1972a,b).

While  $\alpha 3$ -FucT V and  $\alpha 3$ -FucT VI were investigated as recombinant enzymes expressed in CHO cells (Borsig *et al.*, 1996, 1998) little is known on localization, intracellular transport, and release of endogenously expressed fucosyltransferases. Since the major  $\alpha 1,3$ fucosyltransferase activity in human plasma is encoded by  $\alpha 3$ -FucT VI and putatively released from the liver, we examined whether the HepG2 hepatoma cell line expresses  $\alpha 3$ -FucT VI and investigated localization, biosynthesis, intracellular transport, and release of this enzyme in these cells. In addition,  $\beta 4$ -GalT I and  $\alpha 3$ -FucT VI were found to react similarly to monensin treatment which may indicate an analogous post-Golgi pathway (for review, see Dinter and Berger, 1998).

## Results

### Fucosyltransferase activity in HepG2 cells

Lack of fucosylation of liver-derived serum glycoproteins in patients affected by a deficiency of  $\alpha 3$ -FucT VI (Brinkman-

**Table I.** PCR primers for amplifying fucosyltransferases

mRNA species detected	Primers <sup>a</sup>
3-FucT III(585) <sup>b</sup>	U: 5'ACCACTGGGATATCATGTCCAACCTAAGT3' L: 5'GGGCCAGGTCCTTGGGGCTCTGGAAGTCG3'
$\alpha 3$ -FucT IV(589)	U: 5'GGGGCATCCAGGCGCACACTGC3' L: 5'CGCTCGTAGTTGGCACGGTCTG3'
$\alpha 3$ -FucT V(811)	U: 5'CCAGGGCTTATGGCAGTGGAACTGTGCAC3' L: 5'GGGCCAGGTCCTTGGGGCTCTGGAAGTCG3'
$\alpha 3$ -FucT VI(737)	U: 5'ATCCCACTGTGTACCCTAATGG3' L: 5'CGGCAGGAACCTCTCGTAGTTG3'
$\alpha 3$ -FucT VII(448)	U: 5'CCTGGGTGGTCAGCAACTTC3' L: 5'CGGTCACAGATGGCACAGAAAC3'

<sup>a</sup>U, Upper strand primer; L, lower strand primer.

<sup>b</sup>Numbers in parentheses indicates fragment size in base pairs.

Van der Linden *et al.*, 1996) prompted us to investigate  $\alpha 3$ -FucT activity in lysates of HepG2 cells, an established liver carcinoma cell line. To determine the nature of the  $\alpha 1,3$ fucosyltransferase activity detected in HepG2 cells we used the acceptor substrates listed on Table II. Acceptor substrate preference of the overall fucosyltransferase activity was directed toward type 2 acceptors, *N*-acetylglucosamine (LacNAc), and its sialylated derivative 3'-sialyllactosamine (sLacNAc). In addition, type 1 acceptor lacto-*N*-biose as well as type 6 acceptor 2'-fucosyllactose were poorly utilized (The definition of acceptor types are as follows: type 1: Gal $\beta$ 1 $\rightarrow$ 3GlcNAc; type 2: Gal $\beta$ 1 $\rightarrow$ GlcNAc; type 6: Gal $\beta$ 1 $\rightarrow$ 4Glc). The very low ratio of utilization of type 1 to type 2 acceptors allowed to exclude expression of FucTIII to a significant amount. Preference for type 2 acceptor (neutral or sialylated) were in good agreement with the previously reported results of FucT activity in human liver cells (Jezequel-Cuer *et al.*, 1993) and in human serum (Sarnesto *et al.*, 1992). The majority of the  $\alpha 3$ -FucT activity in plasma is due to  $\alpha 3$ -FucT VI which is encoded by the *FUT6* gene (Brinkman-Van der Linden *et al.*, 1996). Indeed, a very similar acceptor specificity profile has been obtained with cloned  $\alpha 3$ -FucT VI expressed in CHO (Borsig *et al.*, 1998), COS cells (Koszdin and Bowen, 1992), or insect cells (De Vries *et al.*, 1997). To assign HepG2 cell-associated  $\alpha 3$ -FucT activity to  $\alpha 3$ -FucT VI or  $\alpha 3$ -FucT V, the enzyme activity of  $\alpha 1,3$ fucosyltransferase from HepG2 cell lysates was subjected to a neutralization experiment with a specific antiserum (designated OLI) raised against recombinant  $\alpha 3$ -FucT VI (Borsig *et al.*, 1998) (Figure 1). The OLI antiserum was able to inhibit the enzymatic activity with LacNAc and sLacNAc as respective acceptors by at least 90%. The inhibition with antibodies was similar with both acceptors suggesting the expression of either  $\alpha 3$ -FucT VI or  $\alpha 3$ -FucT V.

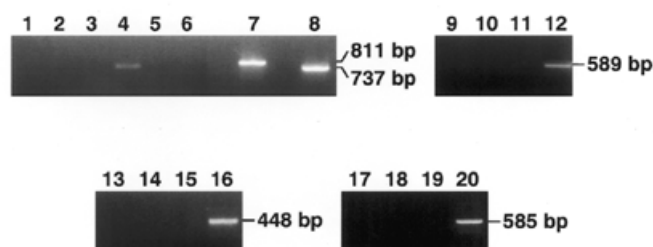
### $\alpha 3$ -FucT VI is the only $\alpha 1,3$ fucosyltransferase expressed in HepG2 cells

Since the OLI antiserum raised against  $\alpha 3$ -FucT VI also cross-reacts with  $\alpha 3$ -FucT V and  $\alpha 3$ -FucT III (Borsig *et al.*, 1998), further determination of the  $\alpha 3$ -FucT expressed in HepG2 cells

**Table II.** Measurement of Fuc-T activity in lysates of HepG2 cells and in the medium; comparison with recombinant α3-FucT VI activity from CHO cells

	Acceptor activity of α1,3fucosyltransferase				
	Relative activity <sup>a</sup> (%)		Activity (pmol/min/mg prot.)		
	rec. α3-FucT VI	HepG2 lysate	HepG2 medium	Lysate	Medium
<i>N</i> -Acetyllactosamine	100	100	100	95	
Galβ1→4GlcNAc					
Lacto- <i>N</i> -biose I	0.3	<0.1	—	<0.1	nd <sup>b</sup>
Galβ1→3GlcNAc					
2'-Fucosyllactose	4.5	3	—	3	nd Fucα1→2Galβ1→4Glc
3'-Sialyl- <i>N</i> -acetyllactosamine	131	110	117	105	2.3
NeuAcα2→3Galβ1→4GlcNAc					

<sup>a</sup>A value of 100% was assigned to the specific activities generated by each enzyme with the acceptor *N*-acetyllactosamine. Relative activities for each of the other acceptors were then calculated by taking the ratio of their respective specific activities to the specific activity observed with *N*-acetyllactosamine;  
<sup>b</sup>Not determined.



**Fig. 2.** RT-PCR of α1,3fucosyltransferases in HepG2 cells. After PCR analysis an aliquot was loaded on a 1.5% agarose gel containing ethidium bromide. α3-FucT V: lanes 1–3, 7; α3-FucT VI: lanes 4–6, 8; α3-FucT IV: lanes 9–12; α3-FucT VII: lane 13–16; α3-FucT III: 17–20; cDNA from HepG2 cells: lanes 1, 4, 9, 13, 17; RNA controls: lanes 2, 5, 10, 14, 18; H<sub>2</sub>O controls: lanes 3, 6, 11, 15, 19; genomic DNA: lanes 7, 8, 12, 16, 20.

was done by RT-PCR analysis. Based on activity measurements, α3-FucT VI and α3-FucT V could be expected. To be able to distinguish between α3-FucT V and α3-FucT VI expression, we used specific primers for α3-FucT V (Borsig *et al.*, 1998) and α3-FucT VI (Table I). Absence of a signal in controls and specific amplification from cDNA and genomic DNA showed expression solely of α3-FucT VI in HepG2 cells (Figure 2, lane 4). PCR amplification with α3-FucT V primers yielded no product (Figure 2, lane 1). RT-PCR amplification with specific primers for α3-FucT III were also negative (Figure 2, lane 17). Although crossreactivity of OLI antibodies with α3-FucT IV and α3-FucT VII was not observed (data not shown), RT-PCR analysis was carried out, also with negative results (Figure 2, lanes 9, 13). Taken together, we conclude that among the five cloned α3-FucTs α3-FucT VI only is expressed in HepG2 cells.

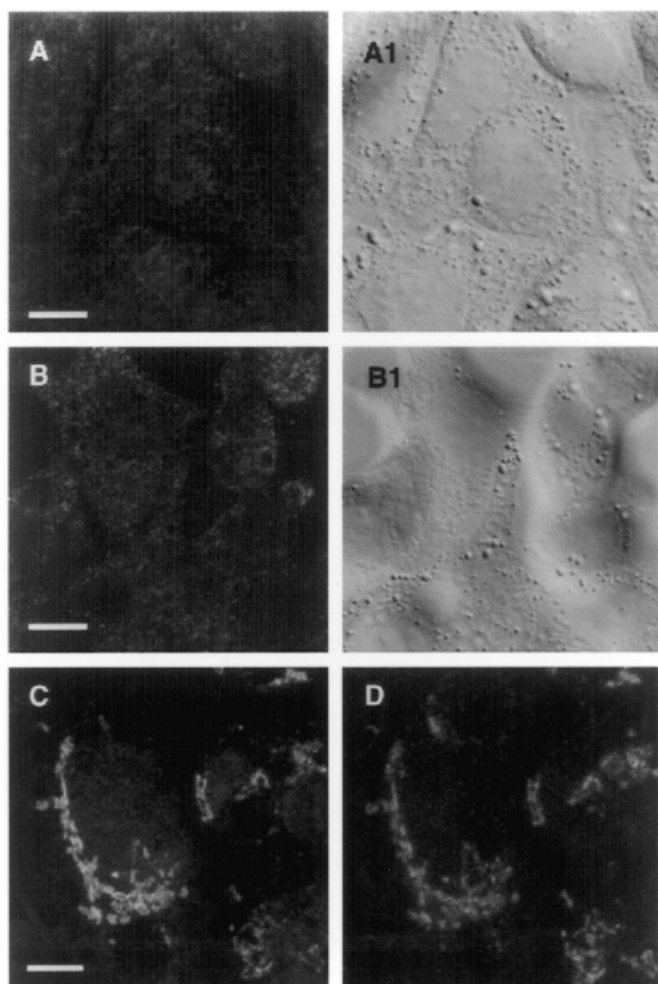
#### α1,3Fucosyltransferase VI is localized to the Golgi apparatus

To determine the steady-state distribution of α1,3fucosyltransferases VI, HepG2 cells were subjected to indirect confocal immunofluorescence microscopy (Figure 3). For staining of α3-FucT VI, previously characterized polyclonal affinity

purified OLI antibodies were used (Borsig *et al.*, 1998) (Figure 3C). A specific Golgi staining was found using OLI antibodies to α3-FucT VI (Figure 3C), while preimmune serum (Figure 3A) or staining with antibodies preabsorbed with α3-FucT VI antigen (Figure 3B) produced background staining only. Golgi location of FucT was further confirmed by double confocal immunofluorescence staining with a monoclonal antibody to β1,4-galactosyltransferase I (Berger *et al.*, 1986) indicating colocalization of both antigens (Figure 3C,D).

#### Maturation of α3-FucT VI in HepG2 cells and its release into the medium

To determine the molecular weight of α3-FucT VI expressed in and released from HepG2 cells, immunoblotting of cell lysates and supernatants with OLI antibodies was carried out (Figure 4). In HepG2 cells, α3-FucT VI appeared as a 46.5 kDa protein, thus slightly smaller than the recombinant enzyme in CHO cells which was detected as a 47 kDa band (Borsig *et al.*, 1998). The small difference might be due to cell-type-specific glycosylation. The enzyme released from HepG2 cells migrated as a 45 kDa protein indicating an analogous processing step for the endogenously expressed enzyme as for the recombinant enzyme described previously (Borsig *et al.*, 1998; Grabenhorst *et al.*, 1998). Fucosyltransferase activity was also measured in the medium (Table II). The specificity of the released enzymatic activity was similar to the intracellular enzyme. The cumulative amount of enzyme released was half of total activity recovered in the cell lysate and in the medium. Maturation of α3-FucT VI in HepG2 cells was analyzed by metabolic labeling followed by immunoprecipitation. HepG2 cells were subjected to pulse-chase analysis (Figure 5). The 43 kDa form (no chase), corresponding to the core glycosylated enzyme, partially shifted to 46.5 kDa after 60 min, which became preponderant after 2 h. The 43 kDa form was sensitive to endo-H treatment (no chase) or PNGase treatment (not shown) and was reduced to a 36.5 kDa form indicating that all four N-glycosylation sites are occupied. After 2 h chase, endoglycosidase-H treatment reduced the mature form to ~45 kDa (chase 120 min) while converting the nonprocessed forms to 36.5 kDa. The

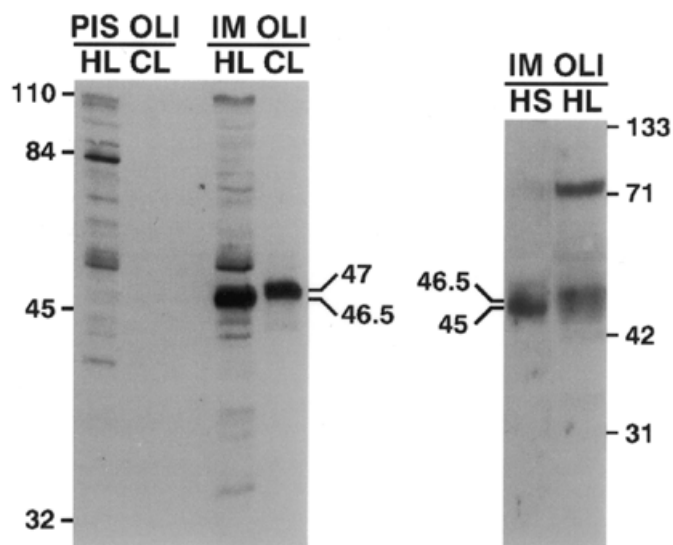


**Fig. 3.** Localization of  $\alpha 3$ -FucT VI in HepG2 cells by confocal immunofluorescence microscopy. Cells were grown and subjected to immunofluorescence labeling using the OLI antiserum as described in *Materials and methods*. (A) HepG2 cells stained with OLI preimmune serum; (A1) corresponding interference contrast picture (Nomarski); (B) HepG2 cells stained with OLI antibodies preabsorbed with sr $\alpha 3$ -FucT VI; (B1) corresponding interference contrast picture (Nomarski); (C) and (D) HepG2 cells double labeling for  $\alpha 3$ -FucT VI using affinity purified OLI antibodies (C) and  $\beta 1,4$ -galactosyltransferase I using the mAb GT2/36/118 (D). Scale bar, 10  $\mu$ m.

partial sensitivity to endoglycosidase most likely indicates that not all of the four N-glycans are converted to complex type. In summary, maturation of  $\alpha 3$ -FucT VI expressed in HepG2 cells was almost identical to the one previously observed for  $\alpha 3$ -FucT VI expressed in CHO cells (Borsig *et al.*, 1998).

#### *Colocalization of $\alpha 3$ -FucT VI with $\beta 4$ -GalT I in monensin-induced swollen vesicles*

Previous data have shown that monensin, an established Golgi-disturbing agent (for reviews, see Mollenhauer *et al.*, 1990; Dinter and Berger, 1998), segregates  $\beta 4$ -GalT I from sialyl-T by relocating  $\beta 4$ -GalT I to peripheral swollen vesicles (Berger *et al.*, 1993). While the nature of these vesicles has not been unequivocally determined, they most probably belong to a post-Golgi compartment. In support of this view,  $\beta 4$ -GalT I

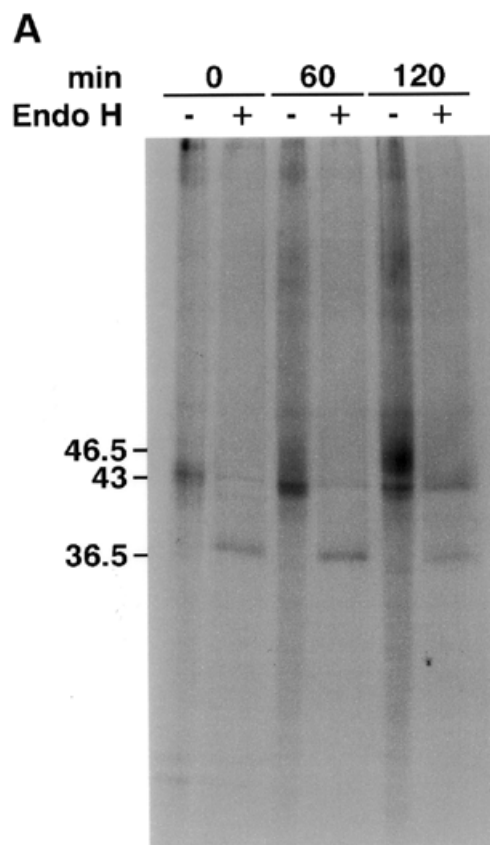


**Fig. 4.** Immunoblotting of  $\alpha 3$ -FucT VI in lysates and supernatants of HepG2 cells. HL, HepG2 cell lysate; CL control lysate from CHO cells stably transfected with  $\alpha 3$ -FucT VI (19); HS, HepG2 cell supernatant, see *Materials and methods*. PIS, OLI stained with OLI preimmune serum; IM, OLI stained with OLI immune serum.

has been found to colocalize with TGN46 in monensin-induced swollen vesicles (Figure 6G/H). Since TGN46 is a well characterized marker of the TGN which recycles to the cell surface within the post-Golgi compartments (for review, see Banting and Ponnambalam, 1997) and which does not colocalize with  $\beta 4$ -GalT I under steady-state conditions (Prescott *et al.*, 1997) the structures in which both TGN46 and  $\beta 4$ -GalT I colocalize (Figure 6 G/H) are compatible with Golgi-derived vesicles. It was therefore of interest to investigate whether  $\alpha 3$ -FucT VI would conform to the same segregative behavior than  $\beta 4$ -GalT I: HepG2 cells were treated with monensin for 30 min and analyzed by confocal immunofluorescence: As shown on Figure 6,  $\alpha 3$ -FucT VI (panel D) colocalizes in swollen vesicles with  $\beta 4$ -GalT I (panel C) in monensin-treated cells, whereas ST6Gal I (panel B) and giantin (panel A), a putative structural protein with predominant cytoplasmic orientation (Linstedt and Hauri, 1993), remain colocalized in a nondisturbed Golgi pattern. The difference between the monensin effect on  $\beta 4$ -GalT I and giantin is shown on Figure 6, E and F, respectively. The monensin-induced dissociation of  $\beta 4$ -GalT I,  $\alpha 3$ -FucT VI, and TGN46 was completely reversible within 1 h after washing-out monensin (not shown). Thus,  $\beta 4$ -GalT I and  $\alpha 3$ -FucT VI, both constitutively secreted enzymes, reacted similarly to monensin treatment but distinctly from ST6Gal I and giantin.

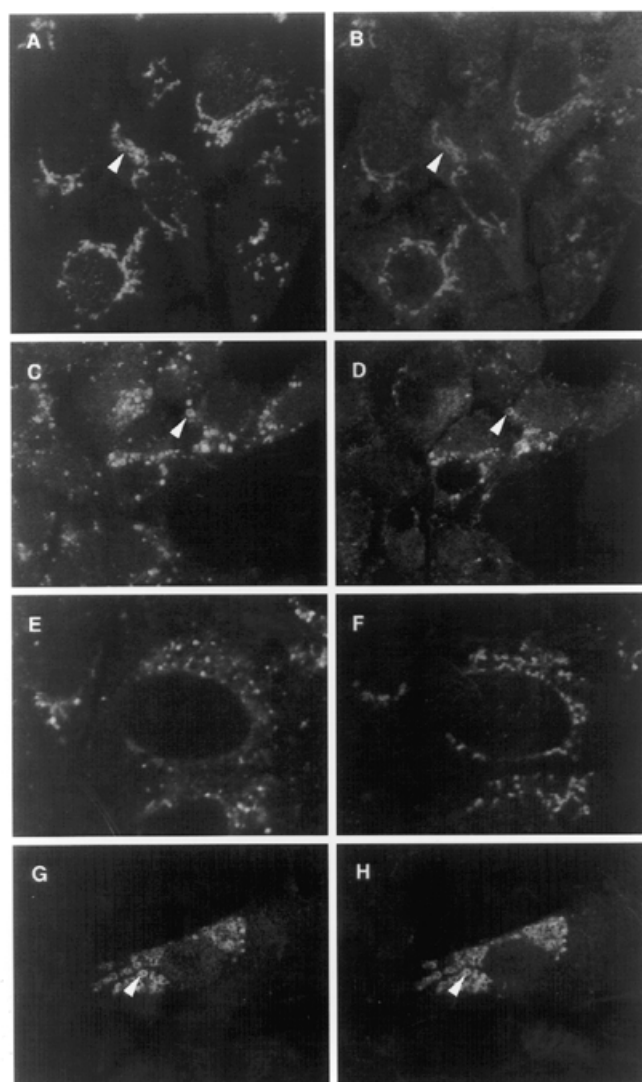
#### **Discussion**

In this work we present the first localization and trafficking study of an endogenously expressed fucosyltransferase. More specifically, this work deals with the presence and expression of  $\alpha 3$ -FucT VI, the product of the *FUT6* gene, in hepatocyte-derived cells. Based on  $\alpha 1,3$ fucosyltransferase activity



**Fig. 5.** Maturation of  $\alpha 3$ -FucT VI in HepG2 cells. HepG2 cells were pulsed for 20 min and chased as indicated, immunoprecipitated, and treated with endoglycosidase H as indicated. Details are described in *Materials and methods*.

measurements in human liver,  $\alpha 3$ -FucT VI was already assumed to be expressed in liver cells (Mollicone *et al.*, 1990; Johnson *et al.*, 1995). Previous work carried out by Johnson *et al.* already surmised expression of  $\alpha 3$ -FucT VI in human liver on the basis of activity measurements, immunochemical evidence using an antibody crossreactive with  $\alpha 3$ -FucT III and Northern analysis of human liver tissue and HepG2 cells. In this work we confirm and extend these findings by RT-PCR analysis of Hep-G2 cell mRNA showing the exclusive expression of  $\alpha 3$ -FucT VI among the five human  $\alpha 1,3$ fucosyltransferases cloned to date. Indeed, enzyme activity in HepG2 cells measured with different acceptors showed a very similar pattern of acceptor substrate preference as already observed by the transient expression of  $\alpha 3$ -FucT VI in COS cells (Koszdin and Bowen, 1992) and its stable expression in CHO cells (Borsig *et al.*, 1998). However, enzyme activity measurements could not unequivocally delineate the number and nature of the possible  $\alpha 3$ -FucTs which are expressed in HepG2 cells. In normal human liver, transcripts of  $\alpha 3$ -FucT V as well as  $\alpha 3$ -FucT VI have been detected (Johnson *et al.*, 1995). In the work of Mollicone and colleagues expression of at least one  $\alpha 1,3$ fucosyltransferase enzyme in liver cells was suggested (Mollicone *et al.*, 1990, 1992). To exclude expres-



**Fig. 6.** Monensin selectively disturbs  $\beta 4$ -GalT I/ $\alpha 3$ -FucT VI structural elements of the Golgi apparatus. HepG2 cells (A–F) and fibroblasts (G, H) were treated with 2  $\mu$ M monensin for 30 min and processed for dual stain immunofluorescence confocal microscopy: (A/B) giantin/ST6Gal I; (C/D)  $\beta 4$ -GalT I/ $\alpha 3$ -FucT VI; (E/F)  $\beta 4$ -GalT I/giantin; (G/H)  $\beta 4$ -GalT I/TGN46. Elements where colocalization is easily apparent are marked with an arrow. Scale bar, 10  $\mu$ m.

sion in HepG2 cells of other members of the  $\alpha 3$ -FucT family, RT-PCR analysis was carried out. After careful adjustment of amplification conditions for each one of the cloned  $\alpha 3$ -FucTs only the expression of  $\alpha 3$ -FucT VI could be documented (Figure 2). Using antibodies specifically recognizing  $\alpha 3$ -FucT VI though crossreacting with  $\alpha 3$ -FucT III and V (Borsig *et al.*, 1998) we identified a band by immunoblotting which likely represents  $\alpha 3$ -FucT VI since the expression of both crossreactive  $\alpha 3$ -FucT III and V has been excluded on the basis of RT-PCR analysis. Moreover,  $\alpha 3$ -FucT V exceeds the size of  $\alpha 3$ -FucT VI by 15 amino acids. Thus, crossreactive  $\alpha 3$ -FucT V would migrate on SDS-PAGE differently from  $\alpha 3$ -FucT VI.  $\alpha 3$ -FucT III could be excluded on the basis of acceptor specificity: lacto-*N*-biose clearly was not a substrate (Table II) as would be expected in the case of  $\alpha 3$  FucT III expression

(Johnson *et al.*, 1995). Here we also assign  $\alpha 3$ -FucT VI to the list of late-acting Golgi-associated glycosyltransferases. Double labeling with  $\beta 4$ -GalT I, a *trans* Golgi enzyme (Slot and Geuze, 1983), by using confocal microscopy, suggests colocalization of both enzymes which would implicate a *trans* localization also for  $\alpha 3$ -FucT VI. Despite several efforts, ultrastructural localization of this enzyme has not been possible. Circumstantial evidence suggested the presence of recombinant  $\alpha 3$ -FucT VI in distal Golgi compartments for its ability to compete with an  $\alpha 2,3$ sialyltransferase (Grabenhorst *et al.*, 1998).

The plasma  $\alpha 1,3$ fucosyltransferase activity is encoded by the *FUT6* gene (Mollicone *et al.*, 1994; Brinkman-Van der Linden *et al.*, 1996). The origin of this activity remains unknown. The liver has been suggested to be one of the potential candidate sources for the plasma activity (Mollicone *et al.*, 1990, 1992; Johnson *et al.*, 1995). The observed release of the  $\alpha 3$ -FucT VI enzyme activity from HepG2 cells provides the first indication that the liver could be, at least in part, the source of plasma  $\alpha 3$ -FucT activity. The 50% of FucT total activity present in medium of HepG2 cells indicates efficient release from the cells. By contrast, pulse-chase analysis showed a rather slow maturation of  $\alpha 3$ -FucT VI, which reached a partial endo-H resistance only after 2 h (Figure 5). Partial endo-H resistance is a common feature for the  $\alpha 3$ -FucT VI enzyme which was already observed in stably transfected CHO cells, where even the secreted form did not reach full resistance (Borsig *et al.*, 1998). Release of  $\alpha 3$ -FucT VI occurs upon proteolytic cleavage accompanied by a reduction in molecular mass of the enzyme. Observations with other glycosyltransferases indicated that release of soluble forms of enzymes occurs by the action of serine-like (Strous and Berger, 1982; Masri *et al.*, 1988; Homa *et al.*, 1993) and cathepsin-like proteases (Weinstein *et al.*, 1987). The site of action of proteolytic processing of released glycosyltransferase remains to be determined. In this regard, it is interesting to observe that the post-Golgi fate of  $\alpha 3$ -FucT VI resembles in several aspects the fate of  $\beta 4$ -GalT I. This enzyme is also easily detectable as a soluble glycosyltransferase in serum (Kim *et al.*, 1972a,b) and other body fluids (Gerber *et al.*, 1979), is located to the *trans* side of the Golgi apparatus (Roth and Berger, 1982; Slot and Geuze, 1983) and appears to share a common post-Golgi pathway with  $\alpha 3$ -FucT VI as inferred by their dissociation from the Golgi apparatus to Golgi-derived vesicles when cells are treated with monensin (Dinter and Berger, 1998; Berger *et al.*, unpublished observations).

The nature of the swollen vesicles induced by monensin treatment has not yet been unequivocally determined. Their appearance and location as well as codistribution of TGN46 are compatible with the view that they are TGN-derived. A number of other genuine Golgi proteins, such as ST6Gal I, giantin (as shown on Figure 6), mannosidase II, *N*-acetylgalactosaminyltransferase II and ST3Gal III (unpublished observations) also remain associated with the Golgi apparatus, indicating a specific post-Golgi behavior of  $\beta 4$ -GalT I and  $\alpha 3$ -FucT VI.

In summary, we show that HepG2 cells harbor and secrete  $\alpha 3$ -FucT VI which is colocalized with  $\beta 4$ -GalT I and which shows a trafficking behavior analogous to  $\beta 4$ -GalT I.

## Materials and methods

### Cell culture and RNA isolation

HepG2 cells were obtained from American Type Culture Collection. They were grown in Dulbecco's modified Eagle medium (Gibco BRL) containing 10% fetal calf serum (complete medium). Total RNA from  $1 \times 10^8$  HepG2 cells was isolated with guanidinium isothiocyanate followed by centrifugation on cesium chloride cushions (Sambrook *et al.*, 1989). The mRNA was isolated from the total RNA using polyT-linked Dynalbeads (Dynal, Norway) according to the manufacturer's protocol.

### RT-PCR analysis of fucosyltransferases

First strand cDNA was prepared using 2  $\mu$ g of poly(A)<sup>+</sup> RNA. Synthesis of cDNA was carried out with 200 U of M-MLV reverse transcriptase (Gibco BRL) and 50 pmol of oligo dT primer. For PCR of fucosyltransferases specific primers were used as depicted in Table I. For  $\alpha 3$ -FucT III 30 cycles were used as follows: 1 min 95°C, 1 min 63°C, 1 min 72°C; for FucT IV 35 cycles were used as follows: 50 s at 95°C; 40 s at 60°C; 50 s at 72°C and final extension of 5 min. For  $\alpha 3$ -FucT V and  $\alpha 3$ -FucT VI 35 cycles were used as described previously (Cameron *et al.*, 1995). For  $\alpha 3$ -FucT VII 35 cycles were used as follows: 50 s at 95°C; 40 s at 58°C; 48 s at 72°C and final extension of 5 min. PCR amplifications with 10 ng of genomic DNA for each FucT to prove the specificity of amplification were carried out. To control for genomic contaminations, each sample was amplified without reverse transcriptase or without DNA. To prove the specificity of PCR fragments, the PCR product was digested by appropriate restriction enzymes (data not shown).

### Immunoblotting

HepG2 cells and CHO 61/11 cells stably transfected with recombinant  $\alpha 3$ -FucT VI were lysed in 1% (w/v) Triton X-100 in PBS. Supernatants were recovered from overnight cultures in serum-free media and concentrated 10-fold prior to analysis. Electrophoresis on 10% SDS/PAGE gel and subsequent immunoblotting was carried out as described previously (Borsig *et al.*, 1998). Nitrocellulose membranes were incubated first with affinity purified OLI antibodies (1:200) followed by goat anti-rabbit horse radish peroxidase (1:5000) and stained using the ECL developing kit according to the manufacturer's instructions (Amersham, UK).

### Fucosyltransferase assay

Cell extracts containing 1% Triton X-100 were prepared as described previously (Borsig *et al.*, 1996). Protein concentrations of cell extracts were determined with a BCA protein assay reagent (Pierce Chemical Co., Rockford, IL). A typical 50  $\mu$ l reaction mixture contained 40 mM sodium cacodylate (pH 6.2), 10 mM MnCl<sub>2</sub>, 10 mM L-fucose, 5 mM ATP, 101  $\mu$ M GDP-fucose (~5000 c.p.m./nmol, mixture of GDP-[U-<sup>14</sup>C] fucose from Amersham and GDP-fucose from Oxford Glycosciences), 5 mM of acceptor substrate (*N*-acetyllactosamine, Lacto *N*-biose I from Sigma, 3'-sialyl-*N*-acetyllactosamine or 2'-fucosyllactose from Oxford Glycosciences) and 30–60  $\mu$ g of protein from cell lysates or 20–30  $\mu$ l of medium. Controls without added acceptor were assayed in parallel under the same conditions. After incubation at 37°C for 2 h the reaction

mix was diluted with cold water and applied to a column containing Dowex 1X8-400, formate form (Kukowska-Latallo *et al.*, 1990). The flow-through fraction, and 2 ml of a subsequent water elution, were collected and counted with 1 volume of Instagel (Packard, IL) in a liquid scintillation counter (Rack-beta 1219, LKB). In the case of octyl-linked acceptors, assays were performed essentially as described previously (Palcic *et al.*, 1989). After stopping the assay with 1 ml of water, the assay mixture was loaded on a C18 Sep-Pak cartridge (Waters), washed three times successively with 5 ml water, and eluted with 5 ml of methanol.

#### Metabolic labeling and immunoprecipitation

HepG2 cells were washed with prewarmed PBS before being starved in methionine-/cysteine-free MEM medium for 20 min at 37°C. The cells were continuously labeled for 1.5 h with 50  $\mu$ Ci [<sup>35</sup>S] methionine/cysteine (EXPRE<sup>35</sup>S<sup>35</sup>S methionine, cysteine labeling mix, NEN/ Du Pont, Wilmington/DE) per ml of met-/cys-free medium or 10 min (pulse/chase) with 100  $\mu$ Ci/ml. Cells were chased for various periods of time with complete DMEM medium and washed 2 times with ice-cold PBS. Cells were scraped off the culture dishes in 10 ml ice-cold PBS containing protease inhibitors per ml: 1  $\mu$ g antipain, 1  $\mu$ g aprotinin, 1  $\mu$ g benzamidin, 0.5  $\mu$ g leupeptin, 1  $\mu$ g pepstatin A, 0.2 mM PMSF, and collected by centrifugation at 1500  $\times$  g for 5 min. Cells were homogenized by passing three times through a 25G<sup>5/8</sup> gauge needle in 10 ml PBS containing 1% (w/v) Triton X-100, and lysed for 30 min at 4°C while rocking. Lysates were cleared by centrifugation for 10 min at 15,000  $\times$  g at 4°C and precleared for 1 h at 4°C with 100  $\mu$ l suspended protein A-Sepharose (Pharmacia) in 10 ml buffer A (PBS, 1% (w/v) Triton X-100). Immunoprecipitation was carried out essentially as described before (Borsig *et al.*, 1998). Controls included preimmune serum and absorption with antigen; in both cases the specific signal was quenched (not shown). For PNGase F and endo-H treatment, 30  $\mu$ l of 0.5% SDS, 1%  $\beta$ -mercaptoethanol in water was added to the washed beads and boiled for 10 min. After cooling, beads were spun down. The supernatant was adjusted to a final conc. of 1% NP-40 (w/v) and incubated for 16 h at 37°C either with 500 U of PNGase F (NEB, Beverly/MA) or with 50 U of endo-H (NEB). For neuraminidase treatment, to the washed beads 30  $\mu$ l of 50 mM sodium citrate, pH 4.5, protease inhibitors (see above) and 50 U of neuraminidase (NEB) were added and incubated for 16 h at 37°C. The reaction was stopped by adding an equal volume of 2 $\times$  SDS-PAGE sample buffer and boiled for 5 min. Immunoprecipitated proteins were separated by SDS-PAGE on a 10% acrylamide gel. After electrophoresis, gels were soaked in 50% methanol/10% acetic acid, dried, and exposed to FUJI x-ray films.

#### Confocal laser scanning double immunofluorescence microscopy

HepG2 cells were fixed and permeabilized as described previously (Borsig *et al.*, 1996). The first antibodies were affinity purified rabbit antibodies to  $\alpha$ 3-FucT VI (OLI) raised to soluble recombinant  $\alpha$ 3-FucT VI (Borsig *et al.*, 1998) or monoclonal antibody mAB2/36/118 to human  $\beta$ 1,4-galactosyltransferase (Berger *et al.*, 1986), respectively. A rabbit polyclonal antiserum to TGN46, the human homologue of rat TGN38 was obtained from  $\alpha$ 3-FucT V. Ponnambalam

(Dundee) Preimmune serum in an appropriate dilution was used. In case of preabsorption of OLI antibodies, affinity purified antibodies were preincubated with 10  $\mu$ g of antigen for 1 h prior to the staining procedure. Fluorescein isothiocyanate (FITC) and Texas red (TR)-conjugated secondary antibodies were obtained from Dako (anti-mouse Ig) and Organon (anti rabbit Ig). For mounting of coverslips embedding medium was used as described previously (Borsig *et al.*, 1996). Immunofluorescence images were taken on a Leica microscope using dual fluorescence mode for Texas red and FITC. Single fluorescence images or extended focus projections were generated using the Imaris software (Bitplane, Zürich Switzerland).

#### Incubation of cells with monensin

Monolayers were incubated for the indicated times in complete medium to which a stock solution of monensin (Calbiochem) in ethanol (1 mg/ml) was added to a final concentration of 2  $\mu$ M monensin (1.4  $\mu$ l/ml medium); controls were supplemented with the same volume of ethanol. Recovery from monensin treatment was carried out by replacing the monensin-supplemented medium by normal medium.

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